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Protein networks and activation of lymphocytes

Ynes A. Helou^a and Arthur R. Salomon^b

^aDepartment of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence RI, 02912, USA

^bDepartment of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912, USA

Abstract

The signal transduction pathways initiated by lymphocyte activation play a critical role in regulating host immunity. High-resolution mass spectrometry has accelerated the investigation of these complex and dynamic pathways by enabling the qualitative and quantitative investigation of thousands of proteins and phosphoproteins simultaneously. In addition, the unbiased and wide-scale identification of protein-protein interaction networks and protein kinase substrates in lymphocyte signaling pathways can be achieved by mass spectrometry-based approaches. Critically, the integration of these discovery-driven strategies with single-cell analysis using mass cytometry can facilitate the understanding of complex signaling phenotypes in distinct immunophenotypes.

Introduction

High-resolution mass spectrometry (MS) has emerged as an attractive wide-scale approach to studying intracellular and extracellular signaling events in lymphocytes. Identification and quantitation of thousands of proteins, as well as their modifications and interaction partners can now be achieved with this approach. Furthermore, the development of novel technologies utilizing MS, such as mass cytometry, has opened up a new age of performing highly parallel single-cell analysis. In this review, we will discuss recent applications of MS technology to unraveling complex signaling networks in lymphocytes.

Shotgun proteomics to interrogate lymphocyte signaling

A comprehensive investigation of protein signaling networks requires the ability to evaluate the dynamic composition of molecular components in time and space. Shotgun proteomics using high-resolution MS provides an unbiased, quantitative, and wide-scale, analysis of complex protein mixtures, delivering snapshots of proteome compositions during cellular

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Corresponding author: Dr. Arthur R. Salomon, Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Box G-E335, Providence, RI 02903. as@brown.edu, Phone: (401) 863-6091 Fax: (401) 863-6087.

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processes. In a typical workflow, proteins extracted from biological samples are enzymatically digested and analyzed using reversed-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Peptides identified using MS are fragmented and sequenced using tandem mass spectrometry (MS/MS), and resulting spectra are analyzed through database search engines to identify the corresponding peptide sequences and proteins. Finally, statistical validation of the data is performed, often through decoy search strategies in which the MS/MS spectra are competitively matched against databases containing both normal and reversed protein sequences to estimate the false discovery rate (FDR) for sequence assignment.

Shotgun proteomics is a powerful approach for quantitation of relative changes in peptide and protein abundance across different cellular states or treatments (Table 1). Both label-free and label-based strategies can be employed for quantitation and have been reviewed in detail elsewhere [1,2]. In LC-MS/MS experiments, MS data is collected for quantitation by the sequential determination of the m/z values and intensities of all peptides eluted into the instrument. Although selection of peptides for fragmentation and MS/MS generation is stochastic and based on the most abundant signals, MS-based quantitation using both label-free and SILAC strategies allows for quantitation in the absence of MS/MS spectra by retention time alignment and accurate mass analysis of MS spectra using information from replicate samples. Importantly, our group has demonstrated the power of this approach by showing that calculating phosphopeptide replicate selected ion chromatogram peak areas using retention time alignment increases the number of replicate peptides quantified 5-fold [3]. Conversely, quantitative information in isobaric mass tag experiments is generated from MS/MS spectra and thus can suffer from ‘missing data’ that may undermine the application of statistical tests.

Shotgun proteomic approaches have benefited investigations of critical signaling pathways in lymphocytes as it has enabled the identification and quantitation of thousands of global alterations in the proteomes of lymphocytes across a diverse range of cellular processes. These MS-based proteomic studies have revealed: (a) insights into proteomic changes occurring in response to various cellular treatments [4–8], (b) unique proteomic signatures across the maturation states of lymphocytes [9,10], and (c) proteins with clinical significance in lymphocyte disease states [11–13]. Another powerful utility of quantitative MS-based proteomics is the interrogation of protein networks within isolated subcellular locations in lymphocytes. Already, the membrane proteomes of primary B cells, NK cells, and T cells have been characterized utilizing this technique on plasma membrane enriched samples [14–16]. Studies have also performed proteomic characterizations of enriched secretory lysosomes from cytotoxic T cells and NK cells [17–19].

Emerging ‘omics’ fields from MS-based proteomics

Harnessing the high-throughput power of MS, investigators have begun the characterization of the secretome and immunopeptidome from signaling lymphocytes. Secreted proteins, which include cytokines, interleukins, and growth factors, are critical intercellular messengers in the immune system, mediating both the communication between effector cells and the orchestration of the immune response. Recently, a quantitative, high-resolution MS

workflow was described to detect and quantify the time-resolved release of proteins from immune cells, the secretome, upon receptor ligation. From this investigation, the authors detected secreted proteins whose abundance increased by a factor of greater than 10,000, illustrating the significant sensitivity and dynamic range of this label-free quantitative approach [20]. Although this particular workflow was applied to macrophages, it will have great utility in interrogating lymphocyte responses to various stimuli. Another recent study implemented quantitative MS along with next-generation sequencing technology to profile the serum antibody repertoire elicited by vaccination, detailing its molecular composition and characteristics [21]. Utilizing similar approaches will be invaluable to defining the serum antibody repertoire and how its diversity and specificity changes in response to diseases.

Another area of research that is enabled by MS is the comprehensive analysis of both the membrane-bound and plasma soluble human immunopeptidome [22]. The immunopeptidome is the collection of thousands of peptides displayed by MHC molecules. Presentation of MHC molecules with their bound immunopeptidomes facilitates the scrutiny of the health-state of cells by circulating T cells. To perform this analysis, peptides bound to immunoaffinity purified MHC are extracted and analyzed by LC-MS/MS. Already, MS-based investigations have revealed the complexity and plasticity of the immunopeptidome and have highlighted critical information on the internal state of the cell that can be gleaned from a system-levels analysis of its composition [23–27].

MS-based investigations of protein-protein interaction networks

Protein-protein interaction (PPI) networks play a critical role in signal transduction in lymphocytes. For example, in T cell receptor (TCR) signaling, scaffolding molecules such as LAT nucleate the assembly of multiprotein complexes that are critical for amplification and diversification of signals that mediate responses resulting from TCR engagement [28]. The study of PPIs has been greatly facilitated by MS technologies, in particular, affinity purification mass spectrometry (AP-MS), which requires the ectopic expression of epitope tags on target ‘bait’ proteins of interest as affinity capture probes. Using this approach, a broad scope of PPI networks can be obtained with high sensitivity, accuracy, versatility and speed. A collection of approaches to define PPIs using AP-MS has been reviewed elsewhere [29–36]. Many of these approaches have been implemented to interrogate PPI networks in lymphocyte signaling [37–40]. Recently, mice bearing a genetic tag that permit AP-MS of signaling complexes containing ZAP-70, LAT, and SLP-76 was described using label-free quantitation. From this system, a membrane-proximal TCR signaling network was identified along with quantitative insights into the temporal regulation of these complexes [39]. A concern for AP-MS experiments is the potential loss of weak interactors. To address this issue, Li et al. implemented an enzyme-generated proximity labeling strategy combined with AP-MS to investigate the molecular composition of B cell receptor (BCR) clusters. In this approach, B cells were treated with HRP-conjugated IgM followed by tyramide-biotin, resulting in biotinylation of proximal proteins. These biotinylated proteins were captured by immobilized streptavidin and analyzed by quantitative MS using SILAC [40].

MS-based investigations of phosphorylation networks in lymphocyte signaling

Post-translational modifications (PTMs) such as phosphorylation, ubiquitination, acetylation, and glycosylation, are critical regulators of signal transduction, enzymatic activity, and protein interactions in immune function. Phosphorylation is the most extensively studied PTM in wide-scale MS-based studies to date as a result of advances in enrichment protocols, MS instrumentation, quantitation strategies, and software tools. Application of MS-based phosphoproteomics approaches has allowed for the unbiased identification and quantitation of thousands of dynamic phosphorylation signaling events in immunological signaling pathways. As we will discuss below, these studies have uncovered a wealth of knowledge on phosphorylation dynamics downstream of immune receptor stimulation.

Our lab as well as other groups have implemented quantitative MS-based phosphoproteomic strategies to perform multi-dimensional comparisons of tyrosine signaling events from mutant and wild type T cells through a time course of TCR stimulation [41–44]. Using this approach, we can accurately quantitate temporal changes in phosphorylation on a large number of sites in response to lymphocyte stimulation (Figure 1). The availability of various mutant clones, as well as the ease of manipulation and scalability of the Jurkat T cell line has made it an ideal model system to interrogate critical pathways downstream of TCR stimulation. The goal of this work is to place the large number of uncharacterized phosphorylation sites revealed in these studies relative to canonical T cell signaling landmarks, providing biological insights into the role of each phosphorylation site. Using this approach, our lab recently demonstrated that pharmacological inhibition of ERK activation in Jurkat T cells resulted in constitutive decreases on the majority of upstream tyrosine phosphorylation sites across a TCR stimulation time course, revealing a critical role for ERK positive feedback in TCR signaling [41]. Various SLP-76 mutant Jurkat cell lines have also been interrogated by our lab using this approach, revealing new modes of phosphoregulation on various critical TCR signaling proteins, including the Src family kinases Lck and Fyn, by SLP-76 [3,42].

A large number of studies have exploited the power of MS-based phosphoproteomics to interrogate a variety of signaling paradigms in lymphocytes. For example, MS-based phosphoproteomic studies have used the Jurkat T cell model to investigate TCR-induced activation events at high temporal resolutions. These studies have highlighted the diverse and dynamic patterns of tyrosine phosphorylation that occur seconds after TCR engagement [45,46]. Furthermore, a comprehensive comparison of tyrosine signaling events triggered by stimulation of different types of receptors was achieved using a similar strategy [47,48]. A large body of literature has implemented the quantitative proteomics workflow to interrogate signaling profiles of various B cell lymphomas [49,50]. Large-scale quantitative phosphoproteomics methods have also been implemented to gain system-wide insights into the role of serine/threonine phosphorylation during lymphocyte activation [51–55].

The application of quantitative phosphoproteomics to interrogate signaling pathways in primary lymphocytes has recently become feasible with the improvement of instrument

sensitivity and mass accuracy, allowing for a physiologically relevant understanding of phosphorylation dynamics in signaling events [49,56–59]. However, limited protein abundance along with the substoichiometric nature of tyrosine site modifications, which is estimated to only make up ~0.5% of the total phosphoproteome [60], has made wide-scale analysis of tyrosine phosphorylation dynamics in primary T cell systems difficult. Nevertheless, studies have interrogated tyrosine phosphorylation events in primary T cells, providing critical insights into tyrosine signaling pathways in a physiological system [56,61].

Statistical analysis of proteomic datasets

A critical requirement for quantifying statistically significant observations in both proteomic and phosphoproteomic datasets is the analysis of at least 3 biological replicates. Many published MS-based studies lack sufficient replicate analyses to perform statistical validation of observations. Furthermore, because of the large number of peptides quantified in these experiments, implementation of multiple testing corrections is required. Corrections such as the family-wise error rate (FWER) or the FDR can be utilized to limit the number of false positive results and have been reviewed extensively elsewhere [62,63]. Our group calculates statistical significance by generating a q-value, the FDR-adjusted p-value, for each replicate observation. A software package called “QVALUE” found on the open-source statistical software R can be used to calculate q-values from p-values [64]. Because of the relative ease of calculating p-values, the software provides an accessible platform to perform this type of robust statistical analysis.

New genetic tools to interrogate signaling pathways

Recent advances in genetic engineering have enabled highly specific perturbations of signaling pathways. In the “analog-sensitive kinase” technology introduced by Kevan Shokat’s group, the ATP-binding pocket of a kinase’s catalytic domain is mutated so that it only accommodates a larger analog of a common non-selective kinase inhibitor [65]. The utility of this technology in interrogating signaling pathways has been demonstrated in recent publications from Arthur Weiss’s group. Interested in understanding the function of basal signaling in T cell activation, they generated mice with an AS allele of Csk in a Csk-deficient genetic background. Using this approach, they confirmed the critical role of Csk in restricting Src family kinase activity in resting primary T cells to maintain basal equilibrium [66]. In another study, this technology was employed to generate mice harboring a ZAP-70 AS allele, which enabled modulation of ZAP-70 kinase activity. Using this system, a sharp TCR signaling threshold for commitment to proliferation was revealed [67]. Engineered AS kinases are powerful tools in which the specificities of critical kinases in lymphocyte signaling pathways can be addressed with minimal off-target effects. Future studies employing this particular model will benefit greatly from phosphoproteomic profiling to determine pathway substrates of kinases within immune signaling pathways that are independent of a potential scaffolding role of the kinase.

MS-based investigations of kinase substrates

Although MS-based phosphoproteomics has facilitated global analysis of phosphorylation networks, this approach in itself is unable to definitively match a given phosphorylation event with an upstream kinase. To address this issue, various groups have designed approaches to screen for kinase substrates using purified active kinases to phosphorylate cell lysates *in vitro*, followed by MS analysis to identify phosphoproteins [68–74]. A major pitfall of these approaches is that they only identify potential kinase substrates *in vitro*. To bridge the gap between *in vitro* phosphorylation and physiological phosphorylation events, Xue et al. introduced a proKALIP approach, in which an *in vitro* kinase assay-based substrate screen was integrated with *in vivo* kinase dependent phosphorylation profiling [75]. Proteins tyrosine phosphorylated *in vitro* overlapping with the kinase-dependent phosphoproteome *in vivo* represented physiological direct substrates. Using this approach, bona fide substrates of the Src protein kinase Syk in B cell signaling were identified [75]. Another approach has recently been developed that addresses the challenges of performing these types of experiments for serine/threonine kinases, which make up roughly 99% of the phosphoproteome and thus present high background in *in vitro* kinase assays. In siKALIP, γ - ^{18}O -phosphoate ATP and moderate dephosphorylation is incorporated into the kinase assay to identify direct kinase substrates in high throughput [76].

Single-cell analysis of lymphocyte signaling

The enormous repertoire and heterogeneity of lymphocytes is well established and plays an important role in generating plastic and diverse responses [77]. Understanding the role of population heterogeneity and the mechanisms by which protein expression levels and PTMs influence cellular signaling and decision-making requires measurements of phenotypic variation at the single-cell level. Seminal work by both Scott Tanner's and Gary Nolan's groups have led to the development of mass cytometry, a technology that couples the single-cell resolution of flow cytometry with the measurement resolution of MS. Antibodies used in this approach are conjugated to metal isotopes that can be differentiated by the mass spectrometer. Because the method is largely unhampered by interference from spectral overlap, it allows for the detection of substantially more simultaneous parameters than traditional flow cytometry (reviewed in [78,79]).

Studies utilizing this approach have revealed that immune cell heterogeneity tends to manifest itself on a continuous scale rather than through distinct populations, underscoring the requirement for greater understanding of signaling pathways on a single-cell level [80,81]. Mass cytometry has also been implemented to investigate the mechanisms utilized by T cells to gauge antigen dose and make the decision to become anergic. The findings revealed that the intracellular balance and signal integration between TCR activating and inhibitory signaling molecules serves as a molecular switch gauging antigen dose [82]. A clear advantage of this technique over traditional MS-based proteomics and phosphoproteomics is the ability to investigate small sample sizes, making it invaluable to the study of primary cells. Despite this advantage, mass cytometry suffers from the lack of unbiased measurements, as well as diminished depth of coverage that is attainable using shotgun proteomics. Furthermore, the subset of modifications or proteins that can be studied

is limited by the availability of antibodies. Nevertheless, implementation of mass cytometry has enabled the quantitation of signal transduction pathways downstream of distinct immunophenotypes, bridging single-cell analysis with a systems-level view of hematopoiesis and immunology in diverse lymphocyte populations. Studies will benefit from a synergistic coupling of the two technologies, where phosphoproteomics identifies a list of candidate sites that can be followed up on with mass cytometry to achieve cellular understanding of bulk assay data.

Conclusions

Unbiased and wide-scale analysis of cellular protein compositions is now achievable with MS-based proteomics technologies, enabling comprehensive examinations into the thousands of signaling events that mediate cellular responses. Investigations into the signaling architecture of lymphocytes in particular has benefitted from recent advances in high-resolution MS. There has been impressive growth in our understanding of the role of phosphorylation in mediating critical signaling events. Additionally, the characterization of dynamic protein interaction networks has been facilitated using MS-based approaches and new 'omics' fields are being developed based on MS technologies. Despite these advances, the field still suffers from a lack of appropriate replicate and statistical analyses, which has markedly diminished the capacity to glean biological conclusions from these datasets. As the field moves forward, it is important that journals implement more stringent requirements for publication of proteomic and phosphoproteomic data, by requiring sufficient biological replicates to ascertain statistical significance.

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- Mass spectrometry (MS) enables the unbiased and comprehensive study of lymphocyte signaling
- New 'omics' fields in immunology are emerging from MS
- Biological replicates are crucial to ascertain statistical significance in MS-based studies

Figure 1. Quantitative phosphoproteomic analysis of canonical TCR signaling proteins
 Label-free heatmaps represent the temporal change in phosphorylation of Jurkat T cells at 0, 3, 5, and 10 minutes of TCR stimulation. Heatmaps were calculated from the averages of five biological replicate experiments. In the label-free heatmaps, black represents peptide abundance equal to the geometric mean for that peptide across all time points. Blue represents peptide abundance less than the mean, whereas yellow corresponds to peptide abundance greater than the mean. A white dot within a label-free heatmap square indicates a statistically significant difference (q value < 0.05) in the fold change in peptide abundance for that time point.

Table 1

Overview of quantitative approaches in MS-based proteomics

	Method	Sample application	Number of comparisons	Quantitation level	Sequencing depth	Quantitative accuracy	Cost
Label-free	Spectral counting	Primary and cultured cells	Many	MS	+++	+	\$
	Ion intensity	Primary and cultured cells	Many	MS	+++	++	\$
Label-based	SILAC	Cultured cells	2–3	MS	++	+++	\$\$
	Isobaric mass tag	Primary and cultured cells	2–8	MS/MS	++	++	\$\$\$